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Association of *CTLA-4* Single Nucleotide Polymorphisms with Autoimmune Hypothyroidism in Iraqi Patients

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Abstract

Genetic and environmental factors are believed to have a key role in the development and pathogenesis of autoimmune thyroid diseases (AITD). This study aimed to investigate the association between two *CTLA-4* gene single nucleotide polymorphisms (SNPs) CT60/rs3087243 and CT61/rs11571319 with autoimmune thyroiditis in a sample of Iraqi patients. Seventy-five patients (67 females, 8 males) and eighty-eight subjects (79 females and 9 males) matched in age, gender, and ethnicity as a control group. Thyroid autoantibodies were present in females more than in males with a total positivity of anti-TPO of 92% and anti-TG positivity of 57.3 %. Thyroid evaluation tests including T3, T4, and TSH were abnormal only in patients not receiving L-thyroxine treatment for autoimmune hypothyroidism, while patients on L-treatment and control subjects had normal results of these tests. *CTLA-4* genotype frequencies were consistent with Hardy-Weinberg equilibrium (HWE) with no significant differences ($p > 0.05$) between the genotypes of patients and the control group. Analysis of *CTLA-4* genotype and allele frequencies in patients and controls indicated the lack of significant differences among these frequencies except for allele *G* of rs3087243 which was associated significantly (P= 0.032) with the disease with OR of 1.62, while allele *A* can have a protective effect with OR of 0.66. Alleles of rs11571319 showed no significant differences despite the decreased frequency of *G* allele (83.33vs.87.5 %) and increased frequency of *A* allele (16.66vs.12.5%) in patients compared to controls. In conclusion, *G* allele of rs3087243 can be considered a risk factor for autoimmune hypothyroidism while no association was found regarding rs11571319 in the Iraqi population.

Keywords: *CTLA-4*; thyroid; susceptibility; autoantibody; case/control study

علاقة تعدد اشكال النيوكليوتيدة الاحادية لمورث CTLA-4 مع مرض قصور الغدة الدرقية المناعي الذاتي لدى المرضى العراقيين

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الخلاصة

تعد العوامل الوراثية و البيئية عناصر مهمة في احداثية امراض الغدة الدرقية المناعية الذاتية. هدفت الدراسة لايجاد المصاحبة ما بين تعدد اشكال النيوكليوتيدة الاحادية لمورث *CTLA-4* و مرض قصور الغدة الدرقية المناعي الذاتي في العراق لعينة من المرضى العراقيين. شملت الدراسة 75 من المرضى (67 اناث و 8 ذكور) و 88 من افراد السيطرة الاصحاء (79 اناث و 9 ذكور) و الذين وافقو المرضى من ناحية العمر و الجنس و العرقية. كشفت النتائج وجود الاجسام المضادة للغدة الدرقية في الاناث اكثر من الذكور و بنسبة 92% للجسم الذاتي المضاد anti-TPO و بنسبة 57.3% للجسم الذاتي المضاد anti-TG و اظهرت النتائج ايضا بان فحوصات تقييم الغدة الدرقية كانت غير طبيعية فقط في مجموعة المرضى الذين لا يتناولون علاج الثايروكسين لقصور الغدة الدرقية. كما أظهر تحليل توازن هاردي-واينبرغ بان الطرز الوراثية لتعدد اشكال النيوكليوتيدة الاحادية للمرضى و افراد السيطرة كانت متناغمة مع التوازن بغياب الفروق ذات الدلالة الاحصائية (الاحتمالية > 0.05) بين التكرارات المشاهدة و المتوقعة للطرز الوراثية. عند تقصي تكرار الطرز الوراثية لتعدد اشكال النيوكليوتيدة الاحادية لمورث *CTLA-4* في المرضى و افراد السيطرة ، لم تكن هناك فروق ذات دلالة احصائية فيما يخص rs3087243 و rs11571319 ، و كذلك الحال من ناحية تكرار الاليلات فقد اظهرت النتائج فروقات غير معنوية ما عدا الاليل *G* ضمن rs3087243 حيث اثبت وجود ارتباط ذو فرق معنوي مع المرض. استنتجت الدراسة بعدم وجود مصاحبة ما بين تعدد اشكال النيوكليوتيدة الاحادية rs11571319 و مرض قصور الغدة الدرقية المناعي الذاتي و وجود مصاحبة للاليل *G* ضمن rs3087243 لدى العراقيين.

1. Introduction

Triiodothyronine (T3) and Thyroxine (T4) are hormones secreted exclusively from the thyroid gland and required by all metabolically active cells; therefore, deficiency or impaired activity of these hormones can result in hypothyroidism which is a common disorder of the endocrine system with a wide range of effects on the body [1].

Hypothyroidism can be presented as Hashimoto's thyroiditis (HT) which is a form of autoimmune thyroid disease (AITD) resulting from T cell-mediated organ-specific immune response against the thyroid gland resulting from immune system deregulation [2].

AITDs affect as high as 5% of the population in general and can also be presented as a form of hyperthyroidism as in Grave's disease (GD). They arise due to the interaction of environmental and genetic factors; primarily the Human-Leukocyte-Antigen DR (*HLA-DR*) gene locus which was the first to be associated with AITD [3]. Other susceptibility genes include thyroid specific genes such as thyroid stimulating hormone receptor (TSHR) and thyroglobulin (TG) as well as immune-modulating genes such as *CTLA-4*, *FOXP3*, *CD40*, *CD25*, *HLA*, specifically *HLA-DR3* which contributes with the highest risk [4].

Autoimmune hypothyroidism or HT has a hallmark of autoantibodies production against the antigenic molecules of the thyroid gland cells; thyroid peroxidase (TPO) and thyroglobulin (TG). These anti-TG and anti-TPO autoantibodies are reported to have a diagnostic value for thyroid diseases [5]. CD8+ cytotoxic T lymphocytes are the leading cause of thyrocyte destruction in HT by antibody-mediated immune processes, with help from differentiated CD4+T helper cells (Th) [6]. The cytotoxic T lymphocyte-associated antigen 4 is critical in negatively regulating T-cell activity. This immuno-regulatory molecule is encoded by the *CTLA-4* gene located on chromosome 2q33 [7]

CTLA-4 gene is expressed in T cells and it had been reported that dysregulation of its expression can lead to several autoimmune diseases including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and type 1 diabetes (T1D)[8]. Other than its expression,

many single nucleotide polymorphisms (SNPs) within its locus have been studied, mainly +49A/G (rs231775), -318C/T (rs5742909), and CT60 (rs3087243) to investigate the association of these SNPs with HT and determine if they can serve as diagnostic biomarkers in a specific population [9]. These SNPs are among the elements that regulate the expression and function of *CTLA-4*, therefore, they can be considered as active factors in the regulation of the immune system due to the impact of this gene on immune system suppression and maintaining self-tolerance. Any breakdown in this tolerance may induce autoimmunity [10,11].

CT60 polymorphism (rs3087243), located in the 3' untranslated region of *CTLA-4*, was considered as a predisposing factor for HT based on Asian population studies of which some confirmed the association of this SNP with HT while others indicated the absence of correlation with HT [12]. This study aimed to explore the status of *CTLA-4* gene polymorphisms, CT60 (rs3087243) and the adjacent SNP CT61 (rs11571319), and their potential association with autoimmune hypothyroidism in Iraqi patients.

2. Materials and Methods

Subjects

Seventy-five unrelated patients with autoimmune hypothyroidism (67 females, 8 males) were enrolled along with eighty-eight age- and gender-matched controls (79 females and 9 males) all with an age range of 20 - 63 years. Patients were selected from the National Diabetes Centre (NDC) / Mustansisiyah University located in Baghdad as they were attending for routine medical care provided by a consultant endocrinologist in this centre from January 2020 to December 2020. Exclusion criteria included history of malignancies, hyperthyroidism, thyroid cancer, and radioactive iodine treatment. All subjects gave informed consent and the study was approved by the ethics committee in College of Science - University of Baghdad.

Blood samples

Five to ten milliliters of venous blood from all patients and the control group were collected by vein puncture using a 10 mL syringe. A volume of 1 mL was transferred into EDTA tubes and was stored at 4°C until DNA extraction within one week. The remaining blood was placed in gel tubes and centrifuged for the separation of serum which was used for thyroid gland evaluation tests. These tests included measurement of the thyroid hormones; T3, T4 and the thyroid stimulating hormone (TSH) by VIDAS system (Biomerieux, France) and detection of anti-TG and anti-TPO autoantibodies by ELISA kits (Aeskulisa, Germany).

Genotyping of CTLA-4 SNPs

The genomic DNA was extracted from whole blood and placed in EDTA using ReliaPrep™ Blood gDNA Miniprep System (Promega, USA) followed by purity and concentration assessment. *CTLA-4* SNPs were located adjacent to each other, so one pair of primers was designed using NCBI Primer-BLAST and supplied by Alpha-DNA Company (Canada). These primers with the following forward sequence: 5'CGGAGTTGTCTTTATCATCC-3' and reverse sequence: 5'-CAGCTGATAGCAACATAGG-3', were used in PCR amplification reaction with a final volume of 30 µl. PCR reaction components included 15 µl Go Taq® Green Master Mix, 0.5 µL of each forward and reverse primers (10 µM), 2 µL DNA sample (100 ng), and 12 µL nuclease-free distilled water. The PCR conditions were programmed as follows after several optimization steps: one cycle of initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 61°C for 1 minute, and extension at 72°C for 1 minute, followed by one cycle of a final extension step at 72°C for 10 minutes. The amplified PCR fragments were detected by electrophoresis in 2% agarose gel to confirm amplification. PCR products were then sent for sequencing by Sanger method using ABI3730XL automated DNA sequencer (Macrogen Corporation – South

Korea). The genotypes and alleles were detected by alignment with a reference sequence from NCBI using Geneious software after.

Statistical Analysis

ANOVA statistical analyses were performed using GraphPad Prism 8 for Windows and IBM SPSS 19.0 for Windows for comparing percentages. Differences were considered to be statistically significant if *P* values were < 0.05. Allele and genotype frequencies were presented as percentage frequencies after testing for agreement with Hardy-Weinberg equilibrium (HWE), and differences were assessed by Pearson's Chi-square test (https://www.genecalculators.net/). The association between *CTLA-4* SNPs and autoimmune hypothyroidism was detected as odds ratio (OR) with the confidence of interval (CI estimate at 95%) (https://www.medcalc.org/calc/odds_ratio.php)

Results and Discussion

In this study, no significant difference (P>0.05) was seen between patients and control groups in regards to gender distribution and mean age which was (50±12.4 years) for patients and (40.87±13.71) for control group. The prevalence of thyroid autoimmunity (TAI) of patients was 9-fold higher in women (n=67 female, 89.3%) compared with that in men (n=8 males, 10.7%). This can be attributed to a combination of female hormones related effects, genetic factors and chromosome X abnormalities [13]. For autoantibodies status, it was found that about half of the patients (49.3%, n=37) were positive for both anti-TG and anti-TPO while the remaining patients were positive for either of them. In total, and as shown in table (1), 92 % (n=69) of patients were positive for anti-TPO with a median of 282.9 (range: 41.6-1154.0 IU/ml) and 57.3 % (n=43) of them were positive for anti-TG with a median of 471.7 (range: 122.3 - 2998.9 IU/ml). Individuals in the control group had either undetectable concentrations of these autoantibodies or their concentrations were below the cut-off values of 40 IU/ml and 120 IU/ml for anti-TPO and anti-TG respectively. Antigenic characteristics of TG and TPO thyroid antigens lead to higher positivity of serum for anti-TPO compared to anti-TG and this adds importance in epidemiological studies where these autoantibodies can be present in apparently healthy individuals who will eventually develop autoimmune hypothyroidism in about 7-9 years after [14].

Table 1- Prevalence of thyroid autoantibodies distributed according to gender

Gender	+ Anti-TPO	-Anti-TPO	+ Anti-TG	-Anti-TG	+ Anti-TPO and + Anti-TG
Males (N=8)	7 (9.33%)	1 (1.33%)	5 (6.6%)	3 (4%)	4 (5.33%)
Females (N=67)	62 (82.66%)	5 (6.66%)	38 (50.6 %)	29 (38.6)	33 (44%)
Total (N=75)	69 (92 %)	6 (8%)	43 (57.3)	32 (42.6)	37 (49.33)
P-value	<0.01	0.102	<0.01	<0.01	<0.01

Thyroid evaluation hormones (T3, T4, and TSH) revealed no significant differences among patients on levothyroxine (L-thyroxine) treatment for chronic autoimmune hypothyroidism (60%, n =45) and healthy controls. As for patients not receiving thyroxine replacement therapy (40%, n=30), results varied significantly (table-2) with lower mean T4 levels and higher mean TSH levels compared to the control group, while T3 did not differ significantly despite being the lowest. L-thyroxine treatment received by chronic autoimmune hypothyroidism patients renders the thyroid evaluation hormones to be in a normal state regardless of the present thyroid autoantibodies. The doses of L-thyroxine varied among patients with a range of 25 – 175 mg depending on several parameters including age and

autoantibodies status. A study on the relationship between thyroid autoantibodies and the dose of levothyroxine found that higher titres of autoantibodies are associated positively with higher dosing of levothyroxine (LT-4) in autoimmune thyroiditis patients [15].

Table 2- concentrations of thyroid function hormones (mean ± SD).

	TSH ± SD (µIU/mL)	**T3 ± SD (nmol/L)**	**T4 ± SD (nmol/L)**
Normal ranges	**0.25 - 5**	**0.92-2.33**	**60 - 120**
Patients on L-thyroxine therapy (n=45)	2.95 ± 0.32 [a*]	1.63 ± 0.69 [a]	92.88 ± 31.48 [a]
Patients without L-thyroxine therapy (n=30)	12.15 ± 4.9 [b]	1.5 ± 0.83 [a]	79.81 ± 33.76 [b]
Control (n=88)	2.84 ± 1.54 [a]	1.71 ± 0.93 [a]	103.16 ± 24.44 [a]
P-value	< 0.01	> 0.05	< 0.01

*Means with the same letter in the same column do not differ significantly (P >0.05).

Before sequencing step, PCR products were subjected to agarose gel electrophoresis to detect the amplified *CTLA-4* gene region. Agarose gel electrophoresis revealed a single band with a molecular size of 508 bp indicating the success of amplification (Figure-1).

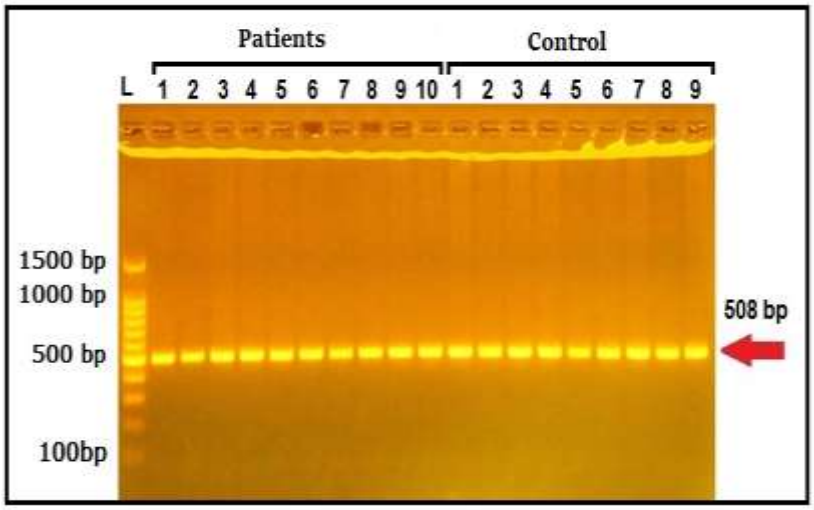

Figure 1-Agarose gel electrophoresis of amplified *CTLA-4* on 2% agarose at 80volt for 60 minutes. Bands of 508 bp appeared in Lanes P1-P10 (samples of patients), and Lanes C1-C9 (samples of controls), while Lane L included a 100bp DNA ladder.

The SNPs CT60 (rs3087243, G>A, T) and CT61 (rs11571319, G>A) were found in two alleles (G and A) and three genotypes (GG, GA, and AA) in both patients and control subjects (Figure-2). Hardy-Weinberg equilibrium (HWE) was achieved in all patients and controls and no significant differences ($p > 0.05$) were discovered between the observed and expected frequencies of these genotypes (Table-3).

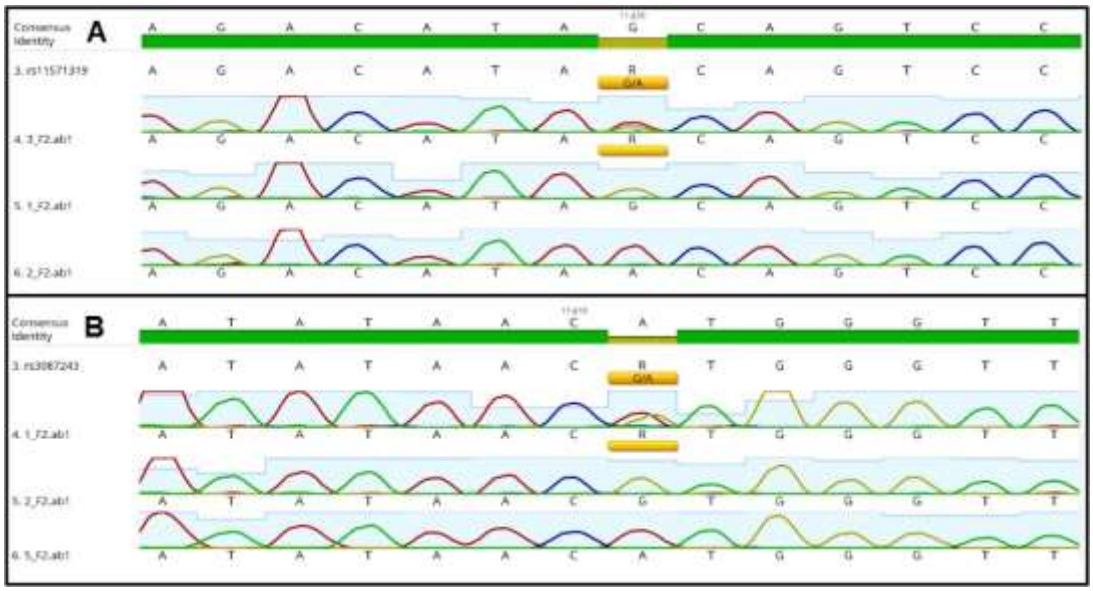

Figure 2-Chromatogram of *CTLA-4* gene sequence showing SNPs (A: rs3087243, G>A, T and B:rs11571319, G>A) and indicating three genotypes: GG, GA, and AA.

Table 3-Observed and expected numbers and percentage frequencies of *CTLA-4* gene polymorphisms CT60 (rs3087243) and CT61 (rs11571319) genotypes and their HWE in HT patients and controls.

SNPs	Genotype	HT Patients (N = 75)				Controls (N =88)			
		Observed		Expected		Observed		Expected	
		N	%	N	%	N	%	N	%
rs3087243	GG	20	26.67	18.75	25	15	17.0	14	15.9
	GA	35	46.67	37.5	50	40	45.5	42	47.7
	AA	20	26.67	18.75	25	33	37.5	32	36.4
	HWE Analysis	X^2=0.33 ; D.F.= 1 ; P= 0.56				X^2= 0.23; D.F.= 1; P=0.63			
rs11571319	GG	51	68.0	52	69.3	67	76.1	67.375	76.6
	GA	23	30.7	21	28.0	20	22.7	19.25	21.9
	AA	1	1.3	2	2.7	1	1.1	1.375	1.6
	HWE Analysis	X^2 0.81= ; D.F.= 1 ; P= 0.36				X^2= 0.13; D.F.= 1; P=0.71			

Inspecting frequencies of detected genotypes and alleles of *CTLA-4* SNPs in patients and controls revealed the absence of significant variations between these frequencies except for *G* allele in rs3087243. The strength of associations between these SNPs and HT risk was measured by ORs with 95% CI as illustrated in Table-4

Table 4-Association analysis between genotypes and alleles of *CTLA-4* gene polymorphisms CT60 (rs3087243) and CT61 (rs11571319) and HT patients and controls.

Genotype or Allele		Patients (N=75)		Controls (N=88)		Odds Ratio	95% Confidence Interval	P-value
		N	%	N	%			
rs3087243	GG	20	26.67	15	17.0	1.77	0.83 to 3.76	0.138
	GA	35	46.67	40	45.5	1.05	0.566 – 1.94	0.877
	AA	20	26.67	33	37.5	0.61	0.31 – 1.18	0.142
	G	75	50 %	70	39.77	1.62	1.04 – 2.52	0.032
	A	75	50 %	106	60.23	0.66	0.42 – 1.03	0.064
rs11571319	GG	51	68.0	67	76.1	0.66	0.33 – 1.32	0.248
	GA	23	30.7	20	22.7	1.5	0.74 – 3.02	0.253
	AA	1	1.3	1	1.1	1.17	0.07 – 19.12	0.909
	G	125	83.33	154	87.5	0.71	0.38 – 1.32	0.287
	A	25	16.66	22	12.5	1.4	0.75 – 2.6	0.287

Statistical analysis revealed that genotype distribution showed no significant differences among subjects; however, allele G of CT60 SNP (rs3087243) was associated significantly (P= 0.032) with the disease and can be conferred as a risk factor with OR of 1.62, while allele *A* can have a protective effect with OR of 0.66. The allele T was not detected in all subjects, indicating its absence in the Iraqi population. These findings agree with a study by Zaletel *et al.*, who also found similar results of association and concluded that this SNP contributes importantly to the production of thyroid autoantibodies [16]. Another study by Ueda *et al*. concluded that allelic variations of this SNP, particularly allele *G*, were responsible for reduced levels of messenger ribonucleic acid of the soluble isoform of *CTLA-4* resulting from alternative splicing owing to the location of this SNP in the non-coding 6.1 kb 3' region [17]. Kavvoura *et al.* reported based on their meta-analysis study that the haplotype *GG* increased the risk of HT by 1.36 fold while for GG and AG combined, the effects were more obvious in Caucasian descent than in Asian descent subjects. Moreover, the total effect of this variant indicated a dose-response effect for the *G* allele depending on the number of copies present in the individual [18]. Ting *et al.* found that allele *G* of CT60 has a significant association with increased risk of Grave's disease in adults and children which is the other form of AITD [19].
These SNPs are among the elements that regulate the expression and function of *CTLA-4*, therefore, they can be considered active factors in the regulation of the immune system due to the impact of this gene on immune system suppression and maintaining self-tolerance. Any breakdown in this tolerance may induce autoimmunity [10,20].
As for CT61 SNP (rs11571319), there was no significant association regarding genotypes and alleles frequency although allele *A* had an OR of 1.4. This SNP was recently reported to be associated with Graves' disease and asthma as well as the most recent novel association with the autoimmune RA risk in Pakistani populations as reported by Aslam [21].
For studying the mode of inheritance, calculations of OR were accomplished on the allele level and the dominant inheritance was clear for allele *G* in CT60 and allele *A* in CT61despite the statistically non-significant differences as shown in the following table:

Table 5-Allele frequencies for CT60 and CT61 and their mode of inheritance

SNPs	Genetic model	Genotypes	patients	controls	OR	95% C.I.	P-value
rs3087243	**Dominant**	GG+GA	20+35	15+40	1.65	0.84 – 3.22	0.142
		AA	20	33			
	Recessive	AA+AG	20+35	33+40	0.56	0.26 – 1.2	0.138

		GG	20	15			
rs11571319	**Dominant**	GG+GA	51+23	67+20	0.85	0.052 – 13.83	0.9
		AA	1	1			
	Recessive	AA+AG	23+1	1+20	1.5	0.753 – 2.99	0.248
		GG	51	67			

Our results are in compliance with Ban *et al.* (2005) on Japanese populations where they suggested that the CT60 *G* allele might act in a recessive fashion since OR was higher in the dominant model than in the recessive model and also suggested the presence of allele dose effect [22].

Conclusion

In conclusion, the SNP rs3087243 is associated with the production of thyroid autoantibodies and the subsequent development of HT and chronic autoimmune hypothyroidism on the allele level where allele *G* might be a risk factor and allele *A* is a protective factor, while SNP rs11571319 is not significantly associated with the disease despite the previous studies that reported its association with the hyperthyroidism form of AITD. To our knowledge, this is the first association study of these SNPs in Iraq.

Conflicts Of Interest

The authors declare that they have no conflicts of interest.

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